Additive activation of hepatic NF-κB by ethanol and hepatitis B protein X (HBX) or HCV core protein: involvement of TNF-α receptor 1-independent and -dependent mechanisms[1]

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SPECIFIC AIM

The present study demonstrates that ethanol activates NF-κB via its metabolite acetaldehyde, which targets a pertussis toxin-sensitive G-protein whereas hepatitis B protein X (HBX) or HCV core protein induces NF-κB by a tumor necrosis factor α receptor 1 (TNFR1) -dependent mechanism in hepatic cells. In view of the essential role of TNFR1 in alcoholic liver injury, targeting TNFR1 by hepatitis viral proteins could contribute to cooperative effects of alcohol consumption and viral hepatitis on liver disease.

PRINCIPAL FINDINGS

1. Ethanol potentiation of HBX- or HCV core protein-activated NF-κB requires its metabolism

To study the effects of ethanol and HBX or HCV core protein on activation of hepatic NF-κB in primary mouse hepatocytes, we used a transient transfection assay with an HBX or HCV core protein expression vector and an NF-κB reporter vector. The results can be summarized as follows: *1)* treatment of primary hepatocytes with ethanol, HBX, or HCV core protein alone markedly activated NF-κB; *2)* treatment of primary hepatocytes with a combination of ethanol and HBX or HCV core protein caused further additive but not synergistic induction of NF-κB. These findings suggest that ethanol and HBX or HCV core protein induce additive activation of NF-κB in primary hepatocytes.

To test whether ethanol potentiation of HBX- or HCV core protein-mediated NF-κB requires its metabolism, HepG2 cells and E47 cells were used. HepG2 cells, which contain no or very low metabolic enzymes for ethanol and its effects should be considered a direct effect; E47 cells, which overexpress CYP2E1, and the effects of ethanol should be regarded as the effects of ethanol metabolism. The results can be summarized as follows: *1)* transfection of HBX induced ~3.8- and 4.2-fold activation of NF-κB in HepG2 and E47 cells, respectively; *2)* transfection of HCV core protein induced ~4.2- and 6-fold activation of NF-κB in HepG2 and E47 cells, respectively; *3)* acute ethanol exposure inhibited basal, HBX-, or HCV core protein-mediated activation of NF-κB in HepG2; *4)* acute ethanol exposure induced ~2.7-fold activation of basal levels of NF-κB and additively but not synergistically potentiated HBX- or HCV core protein-activated NF-κB in E47 cells. These findings suggest that acute ethanol exposure additively potentiates HBX or HCV core protein-activated NF-κB in E47 cells but inhibits it in HepG2 cells

To further confirm the involvement of ethanol metabolism in activation of NF-κB, the ethanol metabolism inhibitor 4-methylprazole (4-MP) was used to block ethanol metabolism. Pretreatment with 4-MP did not affect basal- or HBX-induced NF-κB activity in HepG2 and E47 cells, but markedly blocked ethanol potentiation of basal or HBX-induced NF-κB activity in E47 cells. Taken together, these findings demonstrate that blocking ethanol metabolism attenuates ethanol alone or ethanol plus HBX-induced NF-κB activity, further confirming that ethanol metabolism is involved in ethanol-mediated potentiation of both basal or HBX-induced NF-κB activity in hepatic cells.

2. Acute acetaldehyde exposure additively potentiates HBX- or HCV core protein-activated NF-κB in HepG2 cells, E47 cells, and primary hepatocytes

Effects of acetaldehyde, HBX, and HCV core protein upon activation of hepatic NF-κB were examined in HepG2 cells, E47 cells, and primary hepatocytes. The results showed that acute acetaldehyde exposure in-

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duced ~2.5- to 3.5-fold activation of NF-κB in these three cell lines. A combination of acetaldehyde with HBX or HCV core protein induced ~six- to sevenfold activation of NF-κB in primary hepatocytes, HepG2, and E47 cells. These findings suggest that acute acetaldehyde exposure additively potentiates HBX or HCV core protein-activated NF-κB in primary hepatocytes, HepG2 cells, and E47 cells.

3. HBX, HCV core protein, and acetaldehyde activate NF-κB by an NIK-IKK-IκB-dependent mechanism

Many agents can activate NF-κB; most do so through a common pathway that involves activation of (NIK) and consequent IκB kinase (IKK) and IκB. To study whether the NIK-IKK-IκB signaling cascade was also involved in HBX-, HCV core protein-, or acetaldehyde-activated NF-κB, several dominant negative DNA constructs were used. As shown in **Fig. 1*A***, transfection with dominant negative NIK, IKK, or IκB constructs, but not with wild-type NIK, IKK, or IκB constructs, markedly attenuated HBX-, HCV core protein-, or acetaldehyde-activated NF-κB. These findings suggest that HBX, HCV core protein, or acetaldehyde activates NF-κB by an NIK-IKK-IκB-dependent mechanism.

To further confirm the involvement of IKK and IκB in acetaldehyde-mediated activation of NF-κB, IκB phosphorylation, degradation, and IKK activities were examined. As shown in Fig. 1*B*, a gel mobility shift assay demonstrated that as little as 5 μM of acetaldehyde was able to activate NF-κB. Acetaldehyde rapidly activated NF-κB binding and IκB phosphorylation, followed by degradation of IκB (Fig. 1*B*). Quantitation analyses of these bands with a PhosphorImager showed that treatment of HepG2 cells with 200 μM acetaldehyde for 30 min caused a 10- and 6-fold induction of NF-κB binding and I-κB phosphorylation, respectively (Fig. 1*B*). Kinase assays demonstrated that acetaldehyde activated IKK1 and IKK2 (Fig. 1*C*). Quantitation analyses of these bands with a PhosphorImager showed that acetaldehyde induces ~18- and 5-fold induction of IKK1 and IKK2 activities, respectively (Fig. 1*C*). These findings suggest that acetaldehyde activates IKK-IκB-NF-κB signaling cascade in hepatic cells.

4. HBX or HCV core protein and ethanol activate hepatic NF-κB by a TNF type I receptor (TNFR1) and a PTX-sensitive, G-protein-dependent mechanism, respectively

To test the involvement of TNFR in HBX-, HCV core protein-, or ethanol-mediated activation of hepatic NF-κB, we examined the effects of these stimuli on NF-κB activation in primary hepatocytes from the liver of TNFR1/2 double knockout (TNFR1/2 [−/−]]), TNFR1 (−/−]), TNFR2 (−/−]), and TNFR1/2 (+/+) mice. As shown in **Fig. 2*A***, ethanol, acetaldehyde, TNF-α, HBX, or HCV core protein markedly induced NF-κB activation in TNFR1/2 (+/+) and TNFR2 (−/−]) hepatocytes. Induction of NF-κB by

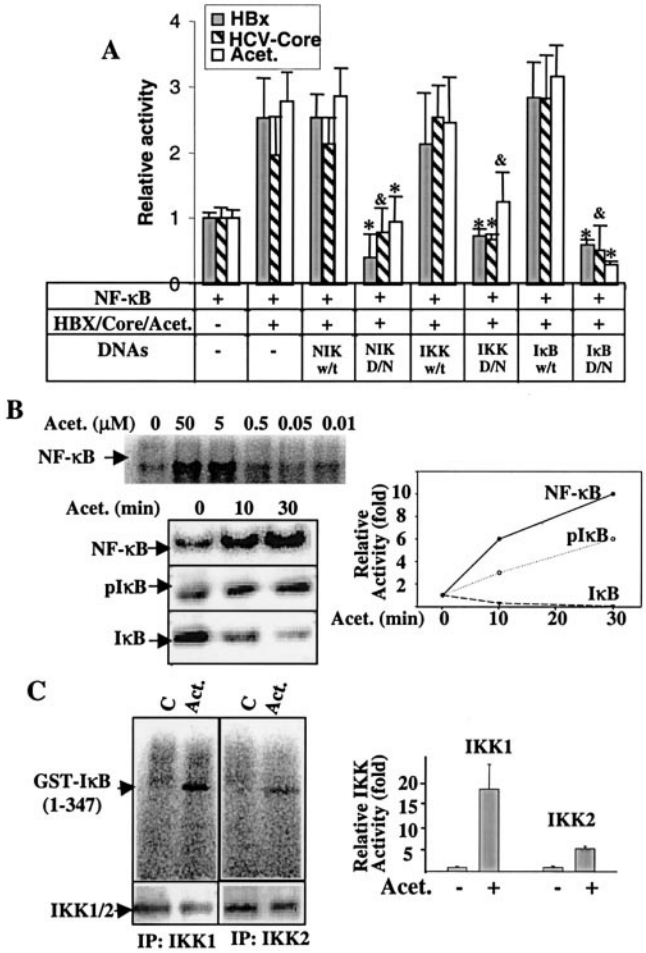

Figure 1. HBX, HCV core protein, and acetaldehyde activate NF-κB by an NIK-IKK-IκB-dependent mechanism. *A)* NF-κB-luc reporter DNA, wild-type, or dominant negative DNA constructs (w/t, wild-type; D/N, dominant negative) and HBX DNA (filled bar) or HCV core protein DNA (hatched bar) were cotransfected into primary hepatocytes. After 4 h, some groups were incubated with 100 μM acetaldehyde (open bar) and cells were cultured an additional 12 h. Luciferase activities were measured. Values shown are means ± SE from a representative of three independent experiments, expressed as fold changes over NF-κB-luc alone transfection control. *$P < 0.01$; $P < 0.05$ vs. NF-κB-luc plus corresponding HBX, HCV core protein, or acetaldehyde group. *B)* Serum-starved HepG2 cells were treated with various concentrations of acetaldehyde for 30 min (top panel) or with 200 μM of acetaldehyde for various times (low panel), followed by detection of NF-κB binding, IκB phosphorylation, and IκB degradation. Acetaldehyde activation of NF-κB binding, IκB phosphorylation, and IκB degradation was quantitated with a PhosphorImager (right panel of Fig. 1*B*). *C)* Serum-starved HepG2 cells were treated with 200 μM of acetaldehyde for 15 min, followed by detection of IKK1 and IKK2. Acetaldehyde activation of IKK1 and IKK2 was quantitated with a PhosphorImager (right panel of Fig. 1*C*).

TNF-α, HBX, or HCV core protein was completely abolished in TNFR1/2 (−/−]) and TNFR1 (−/−]) hepatocytes, whereas ethanol- and acetaldehyde-mediated activation of NF-κB remained unchanged or slightly increased. These findings suggest that ethanol and acetaldehyde activate NF-κB by a TNFR1/2-inde-

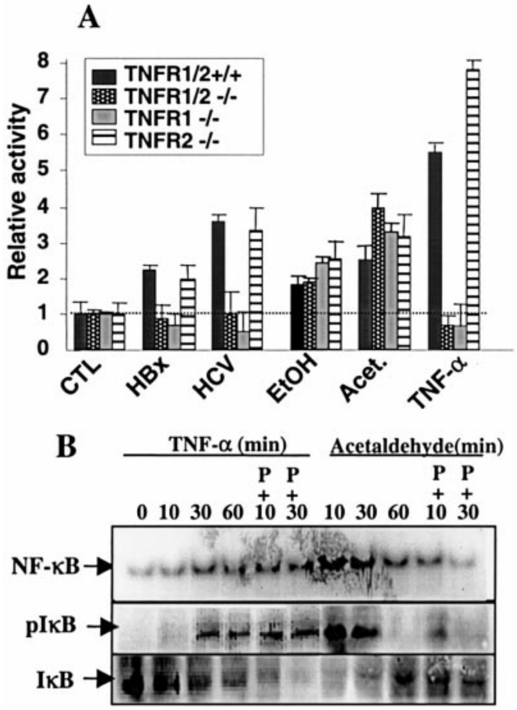

Figure 2. HBX or HCV core protein and ethanol activate hepatic NF-κB by a TNFR1- and PTX-sensitive, G-protein-dependent mechanism, respectively. *A)* NF-κB-luc reporter DNA and HBX or HCV core protein DNA were cotransfected into primary hepatocytes from the liver of TNFR1/2 (+/+), TNFR1/2 (−/−]), TNFR1 (−/−]), or TNFR2 (−/−]) mice. After 4 h, some groups of cells were treated with 100 mM ethanol, 200 μM acetaldehyde (Acet.), or 10 ng/ml TNF-α and cultured an additional 12 h. Luciferase activities were measured. Values shown are means ± SE from a representative of three independent experiments, expressed as fold changes over NF-κB-luc alone transfection control. *B)* Serum-starved HepG2 cells were pretreated with PTX or buffer for 30 min, then stimulated with 200 μM of acetaldehyde or 20 ng/ml of TNF-α for various times, followed by NF-κB binding, IκB phosphorylation, and IκB degradation.

pendent mechanism whereas HBX, HCV core protein, and TNF-α activate NF-κB by a TNFR1-dependent mechanism.

Next we examined whether G-protein was involved in acetaldehyde-mediated activation of NF-κB in hepatic cells. As shown in Fig. 2*B*, pretreatment of HepG2 cells with PTX for 30 min completely abolished acetaldehyde- but not TNF-α-mediated activation of NF-κB, phosphorylation of IκB, and degradation of IκB. These findings suggest that acetaldehyde activates NF-κB by a PTX-sensitive, G-protein-dependent mechanism.

CONCLUSIONS AND SIGNIFICANCE

Although the interaction of alcohol and viral hepatitis has been observed for many decades in the clinic, the underlying mechanisms remain largely unknown. Here we demonstrate for the first time that ethanol and HBX or HCV core protein additively activate the NF-κB signal in hepatic cells and TNFR1 is essential for HBX- or HCV core protein- but not for ethanol-mediated activation of NF-κB, whereas a PTX-sensitive, G-protein-dependent mechanism is involved in acetaldehyde- but not TNF-α-mediated activation of NF-κB. To best interpret these findings, we proposed a model (summarized in **Fig. 3**) that allows for interaction of alcohol and HBX or HCV core protein on NF-κB signaling. HBX or HCV core protein targets TNFR1, followed by NIK-IKK-IκB-NF-κB activation. Ethanol activates NF-κB in the liver via its metabolite acetaldehyde, which targets a PTX-sensitive G-protein, followed by activation of NIK-IKK-IκB-NF-κB. Chronic ethanol consumption induces CYP2E1 expression, which further metabolizes ethanol, followed by potentiation of NF-κB activation. Therefore, alcohol consumption and viral hepatitis infection could cause superactivation of NF-κB in the liver. Activation of this signaling pathway has been shown to play an important role in liver regeneration and anti-apoptosis in the liver, but constitutive or superactivation of NF-κB could cause liver injury by inducing a severe inflammation response or hepatic oncogenesis by stimulating hepatocyte proliferation. Recent evidence has suggested that TNFR1 plays an essential role in alcoholic liver injury; thus, targeting TNFR1 by hepatitis viral proteins could contribute to cumulative effects of alcohol drinking and viral hepatitis on liver disease. FJ

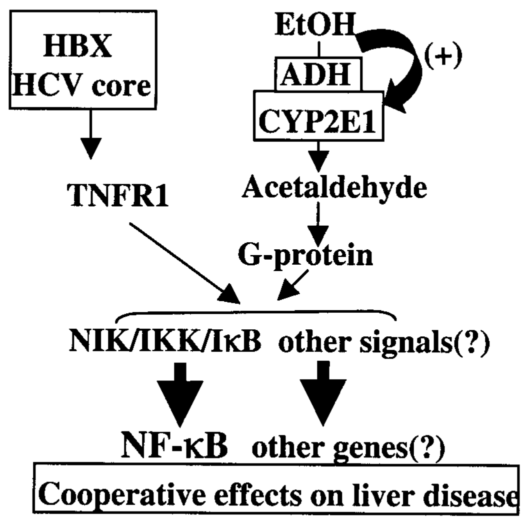

Figure 3. A model for interaction of alcohol and HBX or HCV core protein on NF-κB and consequent induction of cooperative effects on liver disease. HBX or HCV core protein targets TNFR1, followed by activation of NIK, IKK, IκB, and NF-κB. Ethanol activates NF-κB via its metabolite acetaldehyde, which targets a PTX-sensitive G-protein, followed by activation of NIK, IKK, IκB, and NF-κB. Chronic ethanol consumption induces CYP2E1 protein expression, which further metabolizes ethanol, followed by potentiation of NF-κB activation. Ethanol and HBX or HCV also cooperatively activate other signals (such as AP1, SRE). In view of the essential role of TNFR1 in alcoholic liver injury, targeting TNFR1 by hepatitis viral proteins could contribute to cooperative effects of alcohol consumption and viral hepatitis on liver disease.